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Abstract: Epithelial cell adhesion molecule (EPCAM) has recently attained a renewed interest as a candidate protein in diagnosis, prognostication and therapy of various tumor entities. The molecular epidemiology and prognostic relevance of EPCAM in renal cell carcinoma (RCC) and amongst the histological subtypes of RCC is unclear. We analyzed the prevalence and prognostic significance of EPCAM in a tumor tissue micro-array (TMA) composed of 1088 independent RCCs samples by immunohistochemistry (IHC). We found significant variations of EPCAM IHC staining intensities in between the RCC subtypes: In papillary and chromophobe RCC, the majority of tumors (89-93%) showed an at least weak EPCAM protein expression. In the largest subgroup, the clear cell (cc)RCC (n=767), a negative EPCAM IHC was found in 1/3 of the patients and was associated with high-grade disease and nodal metastases. Kaplan-Meier analyses demonstrated a significant association between positive EPCAM IHC and prolonged overall survival, even in a subset of low risk ccRCC. In multivariable analyses, EPCAM represented an independent risk factor of survival throughout all subgroups. For localized, low-grade ccRCC, information of EPCAM IHC raised predictive accuracy of a multivariate model by 5%, compared to T-stage and grade alone. Our findings indicate, that EPCAM is an independent prognostic molecular marker in ccRCC and, especially in localized ccRCC, might be able to provide auxiliary information for a better prognostication.

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Epithelial cell adhesion molecule (EPCAM) is an independent prognostic marker in clear cell renal carcinoma

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Key words:

renal cell carcinoma, epithelial cell adhesion molecule (EPCAM), CD 326, prognosis, molecular marker

Brief description of the impact and novelty of this study:

This to date largest study on the topic tries to settle the question of the significance of EPCAM as a molecular marker in RCC. We found EPCAM independently associated with adverse pathological findings and worse overall survival. Even in the group of localized and low-grade tumors, EPCAM proved its value as an independent prognostic marker. For the first time, we are demonstrating a raise of predictive accuracy by the information of EPCAM protein expression.

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Abstract:

Epithelial cell adhesion molecule (EPCAM) has recently attained a renewed interest as a candidate protein in diagnosis, prognostication and therapy of various tumor entities. The molecular epidemiology and prognostic relevance of EPCAM in renal cell carcinoma (RCC) and amongst the histological subtypes of RCC is unclear.

We analyzed the prevalence and prognostic significance of EPCAM in a tumor tissue micro-array (TMA) composed of 1088 independent RCCs samples by immunohistochemistry (IHC). We found significant variations of EPCAM IHC staining intensities in between the RCC subtypes: In papillary and chromophobe RCC, the majority of tumors (89-93%) showed an at least weak EPCAM protein expression. In the largest subgroup, the clear cell (cc)RCC (n=767), a negative EPCAM IHC was found in 1/3 of the patients and was associated with high-grade disease and nodal metastases. Kaplan-Meier analyses demonstrated a significant association between positive EPCAM IHC and prolonged overall survival, even in a subset of low risk ccRCC.

In multivariable analyses, EPCAM represented an independent risk factor of survival throughout all subgroups. For localized, low-grade ccRCC, information of EPCAM IHC raised predictive accuracy of a multivariate model by ~5%, compared to T-stage and grade alone.

Our findings indicate, that EPCAM is an independent prognostic molecular marker in ccRCC and, especially in localized ccRCC, might be able to provide auxiliary information for a better prognostication.

Introduction

Today, the majority of diagnosed and treated renal cell carcinomas (RCC) represent early stage tumors, assuming a high cure probability after localized therapy. Counter-intuitively, the cancer-specific mortality rates have been rising over the last decades displaying RCC's rather unpredictable nature of disease[1]. Specifically, even in small, low stage tumors, a well acknowledged risk of meta- and synchronous metastatic disease is described[2, 3]. The current version of the EAU guidelines on RCC recommends risk-adapted follow-up schemes after curative treatment, based on TNM classification and Fuhrman grade. There is no general recommendation for integrated prognostic systems or nomograms. Molecular markers are currently not recommended at all, since none of the described markers has been shown to improve predictive accuracy[4]. Yet, aside from diverse risk-stratification tools based on clinicopathologic variables, great hope is set on molecular markers to improve customization of patients´ individual prognostication[5]. Similar to breast- and colon cancer, there is an ongoing debate on the significance of molecular markers to better stratify patients in prognostic subgroups or to trigger potential adjuvant treatment modalities for RCC[6-8].

Epithelial cell adhesion molecule (EPCAM) is a frequently detected marker in (pre-) malignancies of various tissues[9]. In fact, EPCAM was not only the first human tumor antigen identified[10], but also the target of the first monoclonal antibody (mAB) therapy used in clinical oncology[11]. It is broadly overexpressed in premalignant lesions and carcinomas of various origins, such as prostate and bladder, ovarian, lung, colon and breast cancers[12]. Thus, EPCAM IHC is widely used to discriminate neoplasia of epithelial and non-epithelial origin and for the detection and characterization of circulating tumor cells (CTCs)[12, 13].

Controversy persists, whether EPCAM represents a valuable prognostic marker in RCC. While some studies describe an association of loss of EPCAM expression with adverse tumor characteristics and a negative correlation with survival in at least univariant analysis[14, 15], other reports could not support the predictive value of EPCAM protein expression[16, 17].

To address this debate, the aim of this study was to investigate the prevalence and prognostic significance of EPCAM in a large European RCC cohort, using a renal tumor tissue microarray (TMA) including a total of 1088 different tumor samples.

Materials and Methods

Tissue microarray (TMA)

A previously described RCC TMA[18] was used, containing tumor samples from 585 patients operated at the University Medical Center Hamburg Eppendorf (1991-2007) and from 503 patients treated at the University Hospital Basel (1970-1994) due to suspected RCC. The tumors were graded according to the grading system published by Fuhrman et al.[19] and were staged according to the 5th (Basel) and 6th (Hamburg) TNM classification [20 21]. The pT1a and pT1b subclassifications were combined in a global pT1-group, analog the 1997 version. H&E stained histological sections from all paraffin-embedded specimens were reviewed and the tumors were marked on the slides. One 0.6 mm tissue core was punched out from the index area, and transferred into a tissue microarray (TMA) format as previously described by Kononen et al.[22].

Clinical data and follow up

Clinical features (age at time of operation, gender, diagnosis, staging, histological subtype, TNM-stage, grading) were extracted from institutional databases. In case of missing or incoherent values, the corresponding patient charts were retrospectively analyzed, whenever possible.

Follow-up (F/U) data was obtained in accordance with local laws and acquired either from institutional databases or by a standardized postal survey sent to the referring physician or the patient.

Immunohistochemistry (IHC)

Freshly cut TMA sections were stained on one day in a single experiment. High-temperature pretreatment of slides was done in a pressure cooker (pH 6.1 (DAKO buffer, S1699) for 20 minutes. IHC was performed using a monoclonal antibody (1:10, clone VU-1D9, Novocastra, UK) to detect the membrane bound positivity for EPCAM protein.

The Envision system® (DAKO, Glostrup, Denmark) was used to visualize the IHC.

Staining intensities and percentages of positive tumor cells were recorded for each tissue spot by a single pathologist (G.S.). A 4-staged score (0, 1+, 2+, 3+) was deducted from these two parameters according to a previously described scheme[17, 18].

Statistical analysis

For statistical analyses, EPCAM staining was dichotomized into two groups (negative (score 0) vs. positive staining (score 1+, 2+ and 3+). Frequencies of EPCAM staining intensities were analyzed in renal tumors of various histologic subtypes.

In ccRCC the staining intensity of EPCAM was compared to Fuhrman grade, pT-stage, pN-stage (pN0/X vs. pN1/2), and to the absence or presence of synchronous metastases (cM0 vs. cM1). The 2-sided chi-square test was used for comparison of proportions.

The Kaplan-Meier (KM) method was used to explore the prognostic significance of EPCAM staining predicting overall (OS) and cancer-specific survival (CSS) using the log-rank test. The effect of EPCAM staining on survival was evaluated in a multivariate Cox regression model. The concordance (C-) index, which represents the area under the curve adapted for survival data, was determined to estimate and compare the predictive accuracy of multivariate models. The C-index was bias-

corrected by using 200 bootstrap resamples. S-PLUS Professional, version 1 (MathSoft Inc., Seattle, USA) and Statistical Package for the Social Sciences (SPSS) v.12.0 (SPSS Inc., Chicago, USA) were applied. All tests were two-sided with a significance level at 0.05.

Results

Patients and EPCAM Immunohistochemistry

A total of 916 of 1088 tumors were evaluable by EPCAM-IHC. Non-informative cases were either caused by missing tissue on the TMA or absence of unequivocal tumor cells on TMA spots. EpCam immuonstaining was predominantly membranous. Although cytoplasmatic staining was seen, this was nearly always associated with a strong membranous staining. In normal kidney tissue, a strong cytoplasmatic and membranous staining was found in proximal tubular cells.

Among the different RCC subgroups, negative EPCAM expression was significantly more often observed in clear cell carcinomas (ccRCC) than in other histological subtypes of chromophobe (ch)RCC, papillary (pap)RCC and oncocytoma (34% vs. 7-11%; $p<0.001$; Fig.1). As non-ccRCC tumors did not prove a sufficient statistical power due to the low frequency of negative stained cases, all further calculations were performed within the subgroup of ccRCC (n=767). The pathological and follow-up data of the ccRCC-group is summarized in Table 1. The median age at the time of operation was 62 years (range: 15-88), 65% of the ccRCC patients were of male gender.

Association analysis demonstrated that a negative EPCAM staining significantly correlated with a higher Fuhrman grade ($p=0.005$) and with node positive tumors ($p=0.018$; Fig. 2). No significant level was reached for the pT-stage and distant metastasis (M1, $p=0.230$, not shown in Fig. 2).

Survival analyses, all ccRCC

Age ($p<0.001$), but not gender ($p=0.449$), was associated with overall survival in univariate Cox regression analyses. Further, the pTNM stage information and the

Fuhrman grade significantly correlated with OS as determined by Kaplan-Meier analysis (all p<0.001; curves not shown), as did EPCAM expression in the categorized (p=0.001) and the 4-staged model (p=0.014; curves not shown). Figure 3a depicts the Kaplan-Meier curves per dichotomized EPCAM expression for a subset of cases, in which full information for multivariate Cox regression analysis was available (n=441).

For the analysis of CSS, the specific nature of death could be confirmed in 78 out of 249 deceased patients (Tab.1). In 171 cases the specific nature of death could not be determined reliably. In univariate Cox regression analyses, only pT stage (p<0.001), but not Fuhrman grade (p=0.292), pN-status (p=0.056) or EPCAM IHC (p=0.207) achieved significant levels. With the low numbers of confirmed cancer-specific deaths apparently fading the statistical power, further calculations regarding the endpoint CSS had to be omitted.

In a multivariate Cox-proportional hazard model, including nodal positive (pN+) and synchronously metastatic (M1) cases, EPCAM and all other analyzed factors in the model achieved independent predictor status of OS aside from age (all p<0.05; complete data set available in n=441 patients; Tab. 2a). The same was observed when the dichotomized EPCAM IHC information was substituted by the 4-staged variable (p=0.044; data not shown in Tab. 2a).

Non-metastatic tumors

To assess the predictive value of EPCAM IHC in non-metastatic RCC, we selected a subgroup of patients (n=389), excluding cases with synchronous nodal (pN1/2) or distant metastasis (M1) at the time of surgery. In this subgroup the EPCAM protein expression (dichotomized and 4-staged), pT-stage, Fuhrman grade, and age were all independent predictors of OS as determined by multivariate analysis in a Cox-proportional Hazard model (all p<0.05; Tab. 2b for dichotomized EPCAM).

To additionally asses the relative impact provided by the EPCAM IHC, the concordance indices (c-index) of multivariate analyses were compared; the addition of EPCAM IHC did not significantly raise predictive accuracy of a basic model consisting of pT-stage and Fuhrman grade (<1%; 0.6768 vs. 0.6687).

Low/ intermediate risk tumors

A subgroup (n=217) analysis of presumably low/ intermediate risk tumors, excluding high grade (G3/4), pT4, pN+, or M+ cases, was calculated. On univariate analyses, both the dichotomized (p=0.004; Fig. 3b) and the 4-staged (p=0.026) EPCAM IHC categories and Fuhrman grade (p=0.041) significantly predicted overall survival, while pT-stage (p=0.066) did not.

On multivariate analysis including age, pT-stage and grading, younger age and a positive EPCAM expression independently predicted a more favorable OS, while pT-stage and Fuhrman grade did not (Table 2c).

In this subgroup, the addition of dichotomized EPCAM staining into a predictive model, consisting of pT-stage and Fuhrman grade (c-index: 0.5805), resulted in a 5.6% increase of predictive accuracy (c-index: 0.6365).

Low risk tumors analog to UISS

Analog to the histopathological definitions of the UISS risk group stratification by Zisman et al.[23], a low risk subgroup of ccRCC patients (n=138) with pT1 and Fuhrman Grad 1 or 2 only was selected. As information of the preoperative ECOG-Status was missing, we had to assume all patients to have been in a favorable condition (ECOG=0). Consequently, we were not able to further assess the gain of predictive accuracy by incorporation of EPCAM IHC results within the originally defined group.

In this low risk group, EPCAM IHC (p=0.023) but not Fuhrman grade (p=0.184) was a predictor for OS in univariate Cox regression analyses. The 5-year-survival rates for the EPCAM positive and negative group were 85% (standard deviation (SD): 4.6%) and 64% (SD: 8.9%), respectively. The Kaplan-Meier estimates of survival according to dichotomized EPCAM IHC are depicted in Figure 3c. Of note, there was no significant difference regarding the distribution of age between both groups (Mann-Whitney U test: p=0.126)

Discussion

The transmembranous glycoprotein EPCAM – also CD326 – is commonly recognized as a cell-cell-adhesion molecule in normal epithelial tissue. However, recent research revealed that EPCAM is substantially integrated in the processes of cell migration-, differentiation- and proliferation[24, 25]. Here, the activation of the EPCAM glycoprotein leads to intramembranous proteolysis with the release of the **i**ntra**c**ellular **d**omain ("EpICD"). A nuclear translocation of EpICD forms a multiprotein signaling-complex[25]. With a siRNA-induced down-regulation of EPCAM resulting in a decreased cell proliferation, mitogenic influence of this molecule has been further proven[26].

Though, the results of the EPCAM protein expression as a prognostic marker are ambiguous: An overexpression of EPCAM in prostate and ovarian cancer is correlated to a worsen survival[27, 28], and EPCAM siRNA treatment decreased cell migration and invasion by over 90% in breast cancer cells[26]. On the other hand, loss of EPCAM expression in colorectal cancer is associated with the development of metastases and local recurrence[29]. These ambivalent correlations might be due to the multifaceted functions of EPCAM and the multiple pathways involved in EPCAM signaling: On the one side, overexpression of EPCAM results in an up-regulation of

the proto-oncogene c-myc, an elevated metabolic activity and a raised capacity for colony formation, which positively links EPCAM-expression to cell cycle deregulation and proliferation[30]. On the other side, loss of EPCAM is associated with nuclear beta-catenin localization, a reduced cell-cell adhesion, an increased migratory potential and tumor budding in colorectal carcinomas[29].

For RCC the characteristics of EPCAM expression or its loss still have to be defined, as well as the potential role as a prognostic marker.

In analogy to previous studies, we observed significant differences of the EPCAM protein expression between the RCC entities. Especially the chromophobe subtype seems to be characterized by high percentages of EPCAM positive cells, as found in previous (87%[17]; 100%[15]; 100%[31]) and in our study (93%). Since this result in chRCC is in contrast to findings in oncocytomas, Liu et al. suggested that EPCAM-IHC might serve as a differentiator between these entities, which are sometimes hard to distinguish for the pathologist[31].

The predominant group of RCC is composed by ccRCC. For this subtype, Seligson et al. reported a positive correlation between EPCAM expression and low stage and localized diseases[15]. Went et al. could not confirm such a significant correlation, but they described metastatic ccRCC tissue to be significantly less often positive for EPCAM, when compared to the respective primary tumors[17]. Our study demonstrates a significant correlation between low grade and node negative tumors with a positive EPCAM-IHC. In summary, it appears that EPCAM expression is a favorable feature in ccRCC, similar to the findings reported for colorectal carcinomas[29].

This assumption is corroborated by our and previous findings on EPCAM and prognosis: In a series of 193 localized tumors with unreported histologic subtypes, Shvarts et al. described EPCAM to be a predictor of tumor progression as evaluated by univariate analysis[14]. Seligson et al. reported a significant correlation between

EPCAM positivity and CSS for ccRCC, both in uni- and multivariate analyses (n=318)[15]. This group also investigated 150 metastatic ccRCC resected prior to immunotherapy, but the trend for a prolonged survival in tumors expressing EPCAM was not statistically significant[32]. Went et al. could not confirm independent predictor status of EPCAM in IHC of 96 patients, although tumors expressing EPCAM had a trend toward a better survival[17].

Our KM-analyses demonstrate a positive EPCAM status to be significantly correlated with a longer survival. We were able to validate the independent predictor status of EPCAM IHC both in a 4-staged, as well as in a, clinically more practical, dichotomized scoring system. This was especially true for the low (I/II) grade, non-systemic tumors, where EPCAM was the only independent tumor associated factor on multivariate analyses corrected for stage and grade. For this group of patients, which comprises nowadays a large fraction of RCC patients, prognostication is particularly difficult. In our study, the addition of EPCAM raised the predictive accuracy by 4.7% compared to the combination of stage and grade alone. For comparison only, the recently much-noticed prostate cancer gene 3 (PCA3) raises predictive accuracy of a standard biopsy nomogram by 2.3-4.6%[33].

The major limitations of our study are the retrospective design and the lack of sufficient data regarding the specific nature of death, which limits our analyses to the endpoint overall survival. However, as shown in our multivariate analyses, the prognostic significance of EPCAM protein expression was independent of patients' age. Further, the vast majority of the here reported patients were diagnosed years before todays standard therapies for metastatic RCC with multikinase- and m-Tor-inhibitors became available.

The here for the first time described raise of predictive value should be further confirmed in a prospective manner. If confirmed, we suppose this would be an

important step towards the implementation of molecular markers, such as EPCAM, for a more personalized patient prognostication and an individualized follow-up recommendation.

Conclusion

In this large TMA-based study the significant prognostic value of EPCAM in RCC was validated. Beside the differences of EPCAM expression in the RCC subgroups, a significant correlation between EPCAM expression and favorable tumor features was shown for ccRCC. This also reflects the individual patients' prognosis, with EPCAM being an independent predictor of survival, especially in localized diseases of ccRCC.

Figure legends

Table 1: Description of the histopathological features and follow up data of the clear cell RCC subgroup

Table 2a-c: Multivariate analyses: prediction of overall survival for all (a), non-metastatic (b) and low/ intermediate risk (c) ccRCC

Figure 1: Frequencies of EPCAM staining intensities in the 4 largest renal tumor subgroup

Figure 2: Percentages of EPCAM staining intensities in the ccRCC subtype, stratified for pT-stage, nodal status and grade

Figure 3a-c: Kaplan-Meier estimates of overall survival per dichotomized EPCAM IHC: a) all ccRCC; b) intermediate/low risk ccRCC subgroup, excluding pT4, G III/ IV, pN+, or M1 cases; c) analog low risk (UISS) ccRCC subgroup

Picture 1a-d: Samples of negative (a), weak (b), moderate (c) and strong (d) stained EPCAM IHC spots

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ccRCC on TMA (n=767)		n	%
pT	pT1	328	42,8
	pT2	96	12,5
	pT3	331	43,2
	pT4	9	1,2
	pTx	3	0,4
pN	pN0	280	36,5
	pN1/2	46	6,0
	pNx	441	57,5
M status	cM0	715	93,2
	cM+	52	6,8
Fuhrman grade	G1	118	15,4
	G2	280	36,5
	G3	188	24,5
	G4	96	12,5
	n.a.	85	11,1
EPCAM dichotomized	negative	221	28,8
	any staining intensity (+-+++)	436	56,8
	n.a.	110	14,3
EPCAM staining	negative (-)	221	28,8
	weak (+)	103	13,4
	moderate (++)	98	12,8
	strong (+++)	235	30,6
	n.a.	110	14,3
Follow up (F/U) status	dead	249	32,5
	cancer specific death	66	26,5
	other cause of death	12	4,8
	uncertain cause of death	171	68,7
	alive	304	39,6
	no F/U	214	27,9
	median/ mean F/U (months)	34/52	(0.2-354m)

Table 1: Description of the histopathological features and follow up data of the clear cell RCC subgroup

a) uni- and multivariate analyses: all ccRCC (n=441)

	univariate analysis				multivariate analysis			
	p-value	hazard ratio	95% confidence interval		p-value	hazard ratio	95% confidence interval	
			lower	upper			lower	upper
Age	**< 0.001**	1.025	1.012	1.038	**< 0.001**	1.028	1.014	1.041
pT stage	**< 0.001**				**0.022**			
pT 2 vs. 1	0.044	1.589	1.012	2.496	0.074	1.533	0.959	2.452
pT 3 vs. 1	< 0.001	2.346	1.681	3.275	0.005	1.673	1.170	2.393
pT 4 vs. 1	< 0.001	4.611	2.087	10.188	0.036	2.475	1.061	5.778
pN status	**< 0.001**				**< 0.001**			
pN1 vs. pN0	< 0.001	4.946	2.915	8.395	< 0.001	3.171	1.825	5.510
pNx vs. pN0	0.007	1.581	1.133	2.204	0.053	1.409	0.996	1.994
cM1 vs. cM0	**0.002**	2.246	1.339	3.769	**0.004**	2.244	1.301	3.873
Fuhrman grade	**< 0.001**				**< 0.001**			
Fuhrman 2 vs. 1	0.040	1.869	1.028	3.400	0.287	1.396	0.755	2.579
Fuhrman 3 vs. 1	0.001	2.742	1.520	4.948	0.054	1.864	0.989	3.512
Fuhrman 4 vs. 1	< 0.001	6.167	3.347	11.36	< 0.001	3.977	2.071	7.635
EPCAM IHC pos. vs. neg.	**0.001**	0.640	0.486	0.843	**0.022**	0.718	0.541	0.953

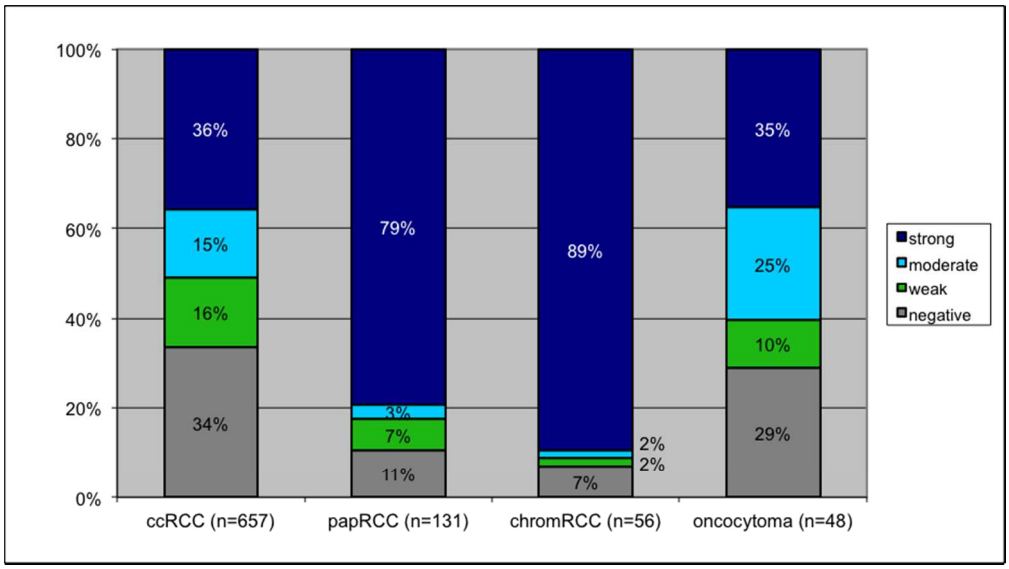
b) uni- and multivariate analyses: non-metastatic ccRCC (n=389)

	univariate analysis				multivariate analysis			
	p-value	hazard ratio	95% confidence interval		p-value	hazard ratio	95% confidence interval	
			lower	upper			lower	upper
Age	**< 0.001**	1.028	1.013	1.042	**< 0.001**	1.029	1.014	1.044
pT stage	**< 0.001**				**0.005**			
pT 2 vs. 1	0.031	1.719	1.051	2.809	0.014	1.892	1.140	3.139
pT 3/4 vs. 1	< 0.001	2.433	1.699	3.482	0.002	1.846	1.256	2.713
Fuhrman grade	**< 0.001**				**< 0.001**			
Fuhrman 2 vs. 1	0.036	2.062	1.048	4.058	0.094	1.797	0.905	3.569
Fuhrman 3 vs. 1	0.002	2.896	1.485	5.650	0.04	2.086	1.034	4.209
Fuhrman 4 vs. 1	< 0.001	6.866	3.436	13.71	< 0.001	5.027	2.437	10.370
EPCAM IHC pos. vs. neg.	**0.001**	0.604	0.447	0.816	**0.004**	0.639	0.47	0.867

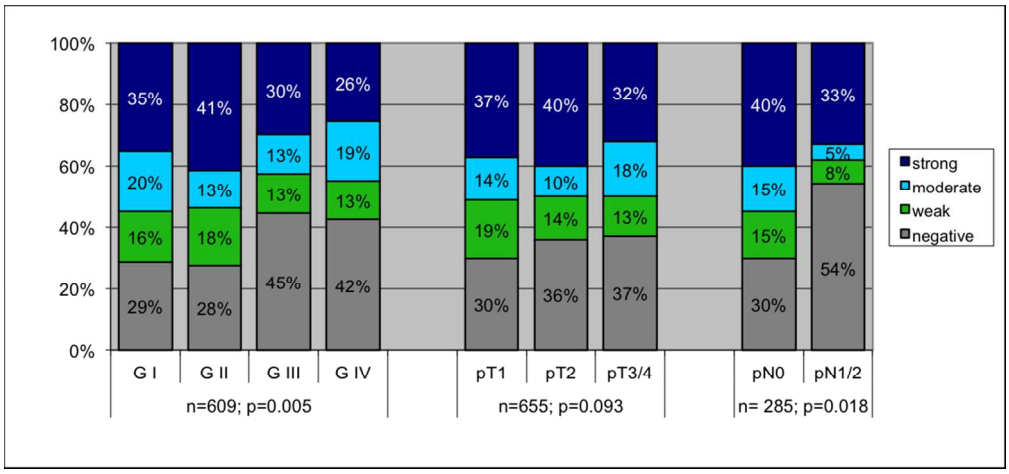
c) uni- and multivariate analyses: low and intermediate risk ccRCC (n=217)

	univariate analysis				multivariate analysis			
	p-value	hazard ratio	95% confidence interval		p-value	hazard ratio	95% confidence interval	
			lower	upper			lower	upper
Age	**0.002**	1.039	1.014	1.065	**0.005**	1.039	1.012	1.067
pT stage	**0.066**				**0.103**			
pT 2 vs. 1	0.749	1.133	0.527	2.435	0.216	1.679	0.739	3.817
pT 3 vs. 1	0.023	1.870	1.092	3.203	0.038	1.809	1.033	3.169
Fuhrman grade 2 vs. 1	**0.041**	2.030	1.028	4.008	**0.091**	1.827	0.908	3.675
EPCAM IHC pos. vs. neg.	**0.004**	0.486	0.296	0.798	**0.010**	0.506	0.301	0.850

Table 2 a-c: Multivariate analyses: prediction of overall survival for all (a), non-metastatic (b). and low/ intermediate risk (c) ccRCC



156x87mm (150 x 150 DPI)



192x89mm (150 x 150 DPI)

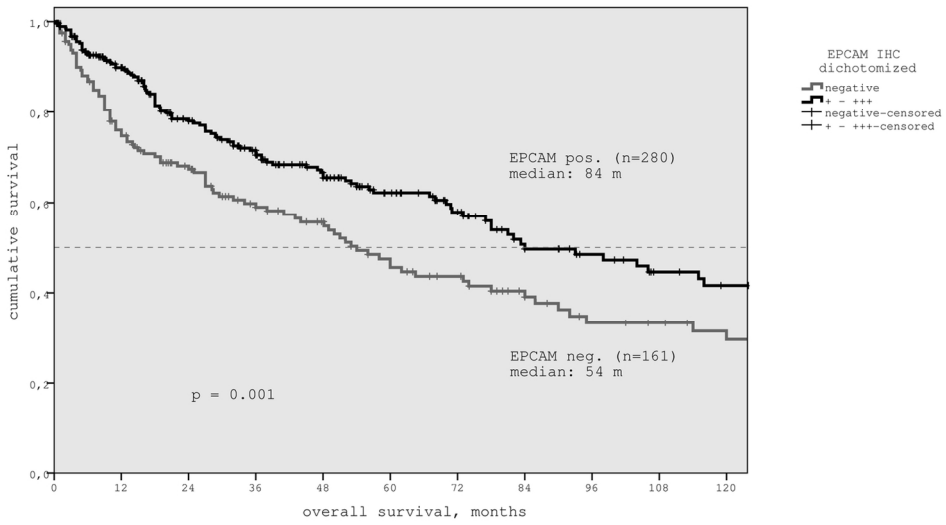
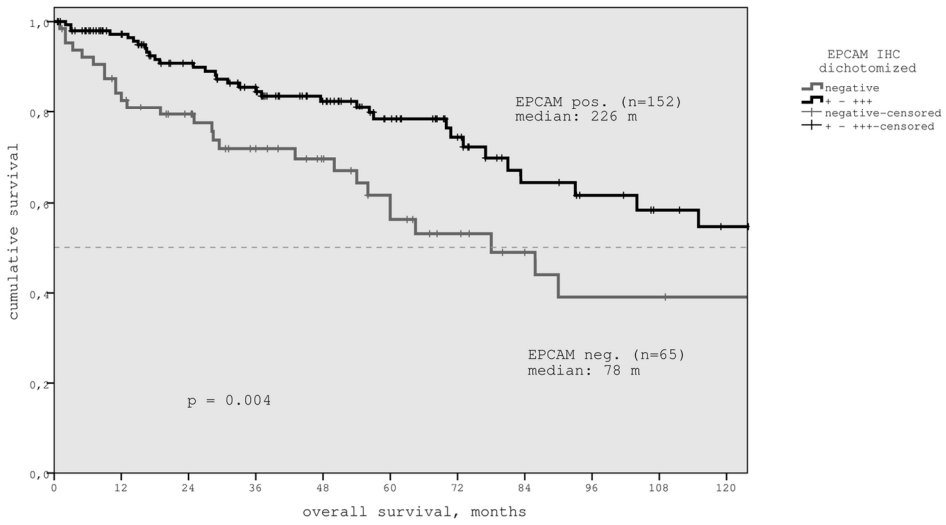
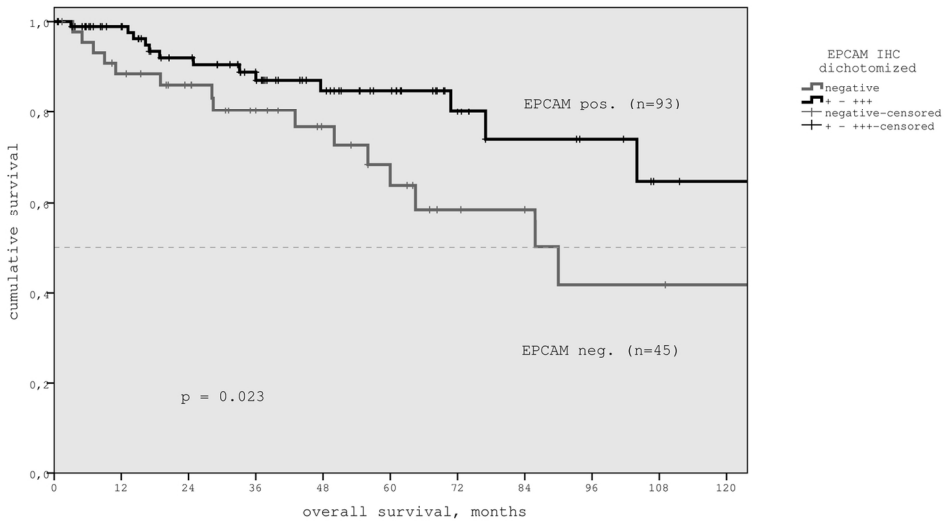


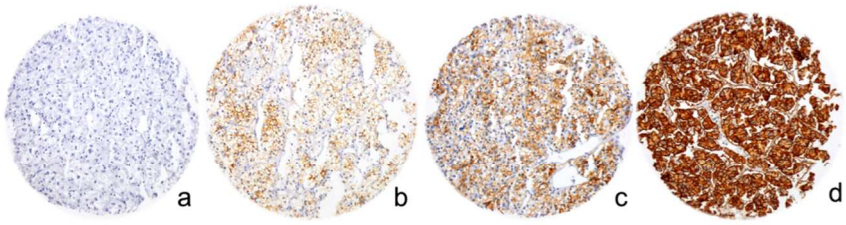
Figure 3a only, 3b and 3c seperately
134x71mm (300 x 300 DPI)



134x71mm (300 x 300 DPI)



134x71mm (300 x 300 DPI)



218x52mm (150 x 150 DPI)